CONFIRMATION OF COCAINE, BENZOYLECGONINE AND COCAETHYLENE BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

1.1 POLICY

This test method may be used to confirm the presence of cocaine (COC), benzoylecgonine (BZE) and cocaethylene (CE) in biological samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

1.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of BZE, COC and CE present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compounds.

1.3 PRINCIPLE

The targeted compounds and internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of solid phase extraction (SPE). Following SPE, the specimens, now termed extracts, are subjected to chemical derivatization. Any BZE present in the samples is converted to the pentafluoropropyl ester, making it suitable for gas chromatography (GC).

Measured volumes of the extracts are injected into a GC where they are separated between a gaseous mobile and liquid stationary phase. Each compound exits the GC at a reproducible time which is termed its retention time.

The GC is coupled to a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, selected-ion-monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any target compound identified in a sample is determined from its calibration curve.

1.4 SPECIMENS

1.4.1 The specimen volume is 1mL.

1.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

1.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; in addition, the specimen may be analyzed at standard volume, as dictated by screening results, to ensure that concentrations of all target compounds present are within the dynamic range of the test method.

1.4.4 Analysis of larger specimen volumes must be approved and documented.

1.5 REAGENTS, MATERIALS AND EQUIPMENT

1.5.1 REAGENTS

1.5.1.1 Acetonitrile (ACN)

1.5.1.2 Ammonium hydroxide (NH_4OH, concentrated)

1.5.1.3 Certified blank blood

1.5.1.4 Deionized water (DI H_2O)

1.5.1.5 Elution solvent

To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store in glass flask/bottle at room temperature and use on date of preparation only. Adjustments to final volume are permitted as long as the proportions of the elution solvent are maintained.

1.5.1.6 Ethyl acetate (EtAC)

1.5.1.7 Hydrochloric acid (HCl, concentrated)

1.5.1.8 0.1M Hydrochloric acid

To 400 mL DI H_2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O. Store the acid in a glass bottle at room temperature for up to 6 months. Adjustments to final volume are permitted as long as the proportions are maintained.

1.5.1.9 Iso-octane

1.5.1.10 Isopropanol (IPA)

1.5.1.11 Methanol (MeOH)

1.5.1.12 Methylene chloride (CH_2Cl_2, dichloromethane)

1.5.1.13 0.1M Phosphate buffer (pH6):

Dissolve 1.7 g Na_2HPO_4 and 12.14 g $NaH_2PO_4 \bullet H_2O$ in 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Check the pH and, if necessary, adjust to 6 ±0.5. Store the buffer in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

1.5.1.14 Pentafluoro propionic anhydride (PFPA)

1.5.1.15 2,2,3,3,3-Pentafluoro-1-propanol (PFPOH)

1.5.1.16 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

1.5.1.17 Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$)

1.5.2 MATERIALS

1.5.2.1 Autosampler vials (glass), inserts and caps

1.5.2.2 Disposable 16 x 100mm tubes

1.5.2.3 Disposable screw-cap or centrifuge tubes with closures

1.5.2.4 Disposable pipette tips

1.5.2.5 Disposable safety closures for 16 x 100mm tubes

1.5.2.6 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206, 200mg/6mL), or equivalent

1.5.2.7 GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 μm film thickness, or equivalent)

1.5.2.8 Laboratory glassware (graduated cylinders, flasks)

1.5.2.9 Volumetric glassware (flasks)

1.5.3 EQUIPMENT

1.5.3.1 Agilent GC (6890 or equivalent)

1.5.3.2 Agilent MS (5973 or equivalent)

1.5.3.3 Calibrated, adjustable piston pipettes

1.5.3.4 Centrifuge

1.5.3.5 Evaporator (Biotage, formerly Zymark, TurboVap)

1.5.3.6 Oven, dry-bath, or wet-bath

1.5.3.7 pH Meter and/or indicating pH paper

1.5.3.8 Vortex mixer

1.5.3.9 Verified, adjustable repeater-pipette

1.5.3.10 Vacuum manifold

1.6 STANDARDS, CALIBRATORS AND CONTROLS

1.6.1 STANDARDS

1.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards (which in turn are used to produce calibrators and positive controls) and the working internal standard.

1.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a. Benzoylecgonine: 1.0 mg/mL
b. Benzoylecgonine-d_3 (BZE-d_3): 1.0 mg/mL
c. Cocaine: 1.0 mg/mL
d. Cocaine-d_3 (COC-d_3): 1.0 mg/mL
e. Cocaethylene: 1.0 mg/mL
f. Cocaethylene-d_3 (CE-d_3): 0.1 mg/mL

1.6.1.3 Working standard (10 ng/μL)

a. Using calibrated pipettes, measure 250 µL each of COC, BZE and CE stock standards into a 25 mL class-A volumetric flask.
b. Add acetonitrile or methanol to the flask to the designated volume.
c. The final concentration of the working standard is 10 ng/µL. The working standard is stored in the freezer in an amber bottle and expires one year from date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

1.6.1.4 Working internal standard (1 ng/μL)

a. Using calibrated pipettes, measure 25 µL each of COC-d_3 and BZE-d_3 stock internal standard, and 250 µL of CE-d_3 stock internal standard to a 25 mL class-A volumetric flask.
b. Add acetonitrile or methanol to the flask to designated volume.
c. The final concentration of the working internal standard is 1 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

1.6.2 CALIBRATORS

1.6.2.1 Calibrators are prepared in certified blank whole blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 1.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure. If the matrix has not been verified as negative, a matrix blank must be included in the batch.

1.6.3 CONTROLS

1.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

1.6.3.2 Positive Controls

a. At least two positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.
d. The control working standard (10 ng/μL) is prepared as described in 1.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 1.7 SAMPLE PREPARATION. Alternatively, quality control personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

1.7 SAMPLE PREPARATION

1.7.1 Label a clean 16 x 100mm tube for each member of the test batch. (i.e., Calibrator, control, case sample)

1.7.2 Place 2 mL of 0.1M phosphate buffer (pH6) into each tube.

1.7.3 Using a calibrated pipette, add 1 mL of certified blank whole blood into each of the six calibrator tubes, the positive control tubes and the negative control tube(s).

1.7.4 Prepare a 1:10 dilution of the working standard. (1 ng/μL)

a. Using calibrated pipettes, combine 0.1 mL of the working standard with 0.9 mL of methanol or acetonitrile in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

1.7.5 Prepare a 1:100 dilution of the working standard. (0.1 ng/μL)

a. Using calibrated pipettes, combine 0.1 mL of the 1:10 dilution with 0.9 mL of methanol or acetonitrile in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

1.7.6 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and prepared dilutions.

Calibrator Description	Volume (μL) Added	Working Standard
Calibrator 1 (10 ng/mL)	100	0.1 ng/μL
Calibrator 2 (25 ng/mL)	25	1 ng/μL
Calibrator 3 (50 ng/mL)	50	1 ng/μL
Calibrator 4 (100 ng/mL)	100	1 ng/μL
Calibrator 5 (500 ng/mL)	50	10 ng/μL
Calibrator 6 (1000 ng/mL)	100	10 ng/μL

1.7.7 Prepare a 1:10 dilution of the control working standard. (1 ng/μL)

a. Using calibrated pipettes, combine 0.1 mL of the control working standard with 0.9 mL of methanol or acetonitrile in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

1.7.8 Using a calibrated pipette, spike the positive controls according to the following table, using the control working standard and the prepared dilution.

Control Description	Volume (μL) Added	Control Working Standard
Control 1 (30 ng/mL)	30	1 ng/μL
Control 2 (750 ng/mL)	75	10 ng/μL

1.7.9 If in-house positive controls are being used, transfer 1 mL of each into their labeled tubes using a calibrated pipette.

1.7.10 Using a calibrated pipette, sample 1 mL of each case sample into its respective tube.

1.7.11 Using a calibrated pipette or verified repeater-pipette, add 100 μL of the working internal standard solution to each tube. Final concentration of the internal standard is 100 ng/mL.

1.7.12 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500 rpm.

1.7.13 Place new, labeled SPE columns into the vacuum manifold.

1.7.14 Condition the SPE columns by passing each of the following solvents completely through under force of gravity.

a. 3 mL methanol
b. 3 mL DI H_2O
c. 1 mL 0.1M phosphate buffer (pH6)

Do not let columns dry out between each conditioning step.

1.7.15 Transfer the contents of each tube to its respective SPE column and allow them to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

1.7.16 Wash the SPE columns by passing each of the following solvents completely through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

a. 3 mL DI H_2O
b. 3 mL 0.1M HCl
c. 3 mL methanol

1.7.17 Dry the columns for 10 minutes under vacuum.

1.7.18 Place clean, labeled screw-cap or centrifuge tubes in the collection rack underneath their corresponding SPE columns.

1.7.19 Pass 3 mL of elution solvent through each SPE column and collect the extracts.

1.7.20 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Extracts must be completely dry for efficient chemical derivatization.

1.7.21 In a fume hood, add 50 µL PFPOH and 50 µL PFPA to each tube and immediately cap. Minimize the time that PFPA is exposed to the atmosphere.

1.7.22 Incubate the tubes for 20 minutes at 55-60°C.

1.7.23 Remove the tubes from heat and cool to room temperature. Alternatively, transfer the tubes to a centrifuge and spin for 2 minutes at 2000 rpm.

1.7.24 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Make sure the extracts are evaporated to dryness before reconstitution.

1.7.25 Reconstitute the extracts by the addition of 50 µL ethyl acetate to each tube. Briefly vortex mix the tubes. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

1.7.26 Transfer the extracts to labeled glass autosampler vials and cap.

1.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation to the date of the test. After entering all vial locations and sample descriptions in the sequence table ensure that the method listing in the table is COCAINE.M for each line.

1.9 DATA ANALYSIS

1.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation.

1.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

1.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves and data analysis parameters (calibration report).

1.9.4 Technical review of the batch is conducted according to the criteria listed below.

1.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the case samples are accepted.

1.10.1 Calibrators and calibration curves

1.10.1.1 Chromatographic peaks for BZE, COC, CE and internal standards shall appear symmetrical (i.e., no co-elution, split peaks, or shoulders).

1.10.1.2 Retention times for target compounds and internal standards shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

1.10.1.3 Quantitative results for BZE, COC and CE in each calibrator shall be within ±20% of their target values with the exception of calibrator 1 which shall be within ±25% of its target. These are inclusive ranges.

For calibrator 1 (target concentration 10 ng/mL), result comparisons will use whole integer values truncated after the first decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL).

For target concentrations ≥10 ng/mL, result comparisons will use whole integer values in units of ng/mL.

1.10.1.4 The calibration curves for BZE, COC and CE shall have correlation coefficients ≥0.99.

1.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

1.10.2 Controls

1.10.2.1 The negative control(s) shall not identify BZE, COC or CE above their respective limits of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

1.10.2.2 Positive controls

a. Chromatographic peaks for BZE, COC, CE and internal standards shall appear symmetrical.
b. Retention times for target compounds and internal standards shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.
c. Quantitative results for BZE, COC and CE in each control shall be within ±20% of their target values. These are inclusive ranges. Result comparisons will use whole integer, truncated results in units of ng/mL.
d. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
e. All positive controls in the batch must meet acceptability criteria for a target compound in order to report quantitative results for that compound in a case specimen.

1.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

1.11.1 Chromatographic peaks for BZE, COC or CE and internal standards shall appear symmetrical.

1.11.2 The retention times for target compounds and internal standards are within ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

1.11.3 The quantitative result for each identified compound must be within the dynamic range of the test method. Results greater than the upper limit of quantitation may be reported qualitatively, provided that all other criteria for acceptance are met.

1.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

1.11.5 If any target compound in a given case sample is outside of the dynamic range it does not invalidate the result for other compounds.

1.12 REPORTING

1.12.1 Results are reported in units of milligrams per liter (mg/L).

1.12.2 The whole integer, truncated results are converted from ng/mL to mg/L.

1.12.3 Converted results are truncated to two significant figures for reporting.

a. For example: benzoylecgonine is measured as 948.72 ng/mL.

b. The unit conversion step truncates the result to 948 ng/mL and then represents the result as 0.948 mg/L.

c. The result is truncated to 0.94 mg/L (two significant figures) and reported.

1.12.4 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

1.13 METHOD PERFORMANCE

1.13.1 Limit of detection: 5 ng/mL (0.005 mg/L)

1.13.2 Lower limit of quantification: 10 ng/mL (0.010 mg/L)

1.13.3 Dynamic range: 10 ng/mL to 1000 ng/mL (0.010-1.0 mg/L)

1.13.4 Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)

1.14 TRACEABILITY

1.14.1 Traceability of the reference materials to SI units is provided through the certificates of analysis provided by the approved reference material supplier.

APPENDIX A
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/ glass wool plug
Temperature	250° C
Split Ratio	2:1
Gas Type	Helium
Gas Saver	Off
Gas Saver Flow	N/A
Gas Saver Time	N/A
Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	3 (Isooctane)
Solvent Wash B	3 (Ethyl acetate)
Sample Pumps	2

Oven / Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.2 mL/min
Initial Temperature	150° C
Initial Time	2.00 min
Ramp Rate	15° C/min
Final Temperature	290° C
Final Time	0.67 min
Transfer Line Temp	280° C

MASS SPECTROMETER

Solvent Delay	6.00 min	MS Quad Temperature	150° C
EM Offset	Set in tune	MS Source Temperature	230° C
Resolution	Low	Dwell Time	50 msec

Signals	Ions	Ion ratios
Benzoylecgonine	300, 421, 316	421/300, 316/300
Benzoylecgonine-d_3	303, 424	424/303
Cocaine	182, 303, 198	303/182, 198/182
Cocaine-d_3	185, 306	306/185
Cocaethylene	196, 317, 272	317/196, 272/196
Cocaethylene-d_3	199, 320	320/199

LIST OF CHANGES

Revision Date	Description	Page Number
09/01/11	Method approved by Washington State Toxicologist. See DRA dated 8/17/11. Method released for use in evidentiary testing on 09/01/11.	All
2/4/16	Added wording for adjustment of prepared volumes in 1.5.1.8, 1.5.1.13, 1.6.1.3 and 1.6.1.4 and clarification to 1.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates in 1.6.1.3 and 1.6.1.4. Edited 1.12.3 to reflect that only two significant figures are used for reporting and added “Printed Copies are Uncontrolled” to footer. Other minor edits throughout.	All
7/10/17	Wording added to 1.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens, and standards throughout SAMPLE PREPARATION in section 1.7. Specified calibrator concentration/ranges in section 1.10.1.3. Edited section 1.10.2.2.e to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-9